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(71) Applicant *(for US only)*: KAISER, Bonnie, Lu (executrix for the deceased inventor) [US/US]; 515 East 79th Street, Apartment 28E, New York, NY 10021 (US).

(71) Applicant *(for all designated States except US)*: THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES, INC. [US/US]; 10280 North Torrey Pines Road, La Jolla, CA 92037 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : GHOSH, Soumitra, Shankar [IN/US]; 13285 Deron Avenue, San Diego, CA 92129 (US).

(72) Inventor: KAISER, Emil, Thomas (deceased).

(74) Agents: WATT, Phillip, H. et al.; Fitch, Even, Tabin & Flannery, Room 900, 135 South LaSalle Street, Chicago, IL 60603 (US) et al.

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(57) Abstract

Inhibitors of the Zn^{+2}-metalloendopeptidases, enkephalinase, angiotensin-converting enzyme, and collagenase are provided. The enkephalinase inhibitors of the invention are useful as analgesics or antihypertensives. The angiotensin-converting enzyme inhibitors of the invention are useful as antihypertensives. The collagenase inhibitors of the invention are useful in treating diseases, such as corneal ulceration, periodontal disease, and arthritis, which involve undesirable activity of bacterial or mammalian collagenases. The inhibitors of the invention are peptide or peptide-ester derivatives of the dipeptide analogs of the formula X_1-$(CR_3R_4)_z$-$(CR_5R_6)_x$-CHR_9-CO_2H wherein X_1- is a functional group, such as $(N=C)$ (CH_2) $(C=O)$-, from which a Zn^{+2} metalloendopeptidase, at its active site, is capable of abstracting a proton to yield and activated functional group capable of forming a stable, covalent bond with a residue in the active site; z is 0 or 1, wherein, when z is 0, the group -$(CR_3R_4)_z$-$(CR_5R_6)_x$- is not in the compound and the group X_1-is bonded directly to the group -(CHR_9)-; x is 0 if z is 0 or is 0 or 1 if z is 1, wherein, when x is 0 and z is 1, the group -$(CR_5R_6)_x$- is not in the compound and the group -(CR_3R_4)- is bonded directly to the group -(CHR_9)-; R_2, R_3, R_4, R_5, and R_6 are independently hydrogen or alkyl of 1 to 3 carbon atoms; and R_9 is benzyl, alkyl of 1 to 5 carbon atoms, or hydrogen. Among the dipeptide analogs derivatized with peptides or peptide esters in accordance with the invention is 2-benzyl-5-cyano-4-oxopentanoic acid, which the invention provides substantially free of its 3- benzyl regioisomer.

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DIPEPTIDE-ANALOG-BASED METALLOENDOPEPTIDASE INHIBITORS AND METHODS OF USING SAME

TECHNICAL FIELD

This invention relates generally to inhibitors of metalloendopeptidases and more specifically to inhibitors of enkephalinase, angiotensin-converting enzyme, and collagenase and therapeutic uses of said inhibitors.

BACKGROUND OF THE INVENTION

Enkephalins, Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), are pentapeptides which specifically bind opiate receptors in the brain and thereby are involved in regulation of nociceptive or pain stimuli. The enkephalins are generally short-lived molecules, being rapidly hydrolyzed into inactive fragments following their synaptic release.

A variety of peptidases are known which are able to cleave enkephalins, in vitro, into biologically inactive fragments. Cleavage by an aminopeptidase results in release of the N-terminal tyrosine. A dipeptidylamino-peptidase has been implicated in the cleavage of the Gly^2-Gly^3 bond. The Zn^{+2} metalloendopeptidases, enkephalinase (EC 3.4.24.11, also known as "neutral endopeptidase 24.11") (hereinafter referred to as "enkephalinase") and angiotensin-converting enzyme (EC 3.4.15.1, also known as "angiotensin I converting enzyme")(hereinafter referred to as "angiotensin- converting enzyme" or "ACE") cleave the Gly^3-Phe^4 bond.

It is widely accepted that enkephalinase is the enzyme primarily responsible for the in vivo hydrolytic cleavage of enkephalins and, as such, has a significant role in causing and regulating pain. Competitive inhibitors of enkephalinase are known which are active as antinociceptive agents (i.e., pain-relievers or "analgesics") in vivo in mammals, including humans. See, e.g., Erdos and Skidgel, FASEB J. 3, 145 (1989); Grazia et al., Eur. J. Pharmacol. 125, 147 (1986).

Because enkephalinase is also known to proteolytically cleave, and thereby inactivate, the circulating form, ANF(99-126), of atrial natriuretic factor (ANF), enkephalinase is thought to have a role in regulation of fluid balance and blood pressure. Indeed, enkephalinase inhibitors, by inhibiting the degradation of ANF(99-126), might be employed in vivo to induce fluid and Na^+ excretion and reduce blood pressure. Increases in urine volume and Na^+ secretion are potentiated by, for example, the potent enkephalinase inhibitor thiorphan. See Erdos and Skidgel, supra.

Though, like enkephalinase, ACE cleaves enkephalin at the Gly-Phe bond, ACE's low affinity for enkephalins (K_m~1 mM) and relatively low rate of hydrolysis rule it out as a significant enzyme in the inactivation of endogenous enkephalins. ACE plays a significant role in blood pressure control, as the enzyme is primarily responsible for the conversion of the decapeptide angiotensin I, by proteolytic cleavage of the Phe^8-His^9 peptide bond, to the octapeptide angiotensin II, a potent vasoconstrictor. See, e.g., Erdos, Lab. Invest. 56, 345 (1987); Ondetti and Cushman, Ann. Rev. Biochem. 51, 283 (1982); Ehlers and Riordan, Biochemistry 28, 5311 (1989).

Competitive inhibitors of ACE, including captopril, enalaprilat and lisinopril, are used in vivo to reduce hypertension in humans.

Collagenases are Zn^{+2} metalloendopeptidases involved in the turnover, remodeling or degradation of collagen and have been isolated from numerous species, from bacterial to human. The substrate specificities of collagenases vary, although they all proteolytically cleave a peptide bond in a collagen. The collagenases of Clostridium histolyticum (EC 3.4.24.3) catalyze cleavage of the X-Gly bond in the repeating sequence -Gly-Pro-X-Gly-Pro-X- of collagen, where X is frequently Ala or Hyp but may be any amino acid. The collagenase of Achromobacter iophagus (EC 3.4.24.8) catalyzes cleavage of the X-Gly bond in X-Gly-Pro-Y

sequences. Lecroisey and Keil, Biochem. J. 179, 53 (1979). Mammalian collagenases have a recognition sequence of at least five amino acids and proteolytically cleave the Gly-Ile or Gly-Leu peptide bond in the sequence Pro-(Non-Pro)$_1$-Gly-(Ile or Leu)-(Non-Pro)$_2$. In most cases of mammalian collagenases that have been characterized, the amino acid on the carboxyl side of the scissile bond is Ile, (Non-Pro)$_1$ is Leu or Gln, (Non-Pro)$_2$ is Ala, and there is an additional Gly at the amino terminal end and an additional Gly at the carboxy-terminal end of the pentapeptide, minimal recognition sequence. See Johnson et al., J. Enzyme Inhibition 2, 1 (1987).

Inhibitors of collagenase are thought to have a number of therapeutic applications, including treatment or inhibition of periodontal disease, via inhibition of both bacterial and human collagenases implicated in the disease; treatment or inhibition of collagen-degradative effects of bacterial infections, arising from bacterial collagenase activity; treatment of corneal ulceration that is caused, at least in part, by collagenase-catalyzed collagen degradation; treatment of arthritis, including rheumatoid arthritis and osteoarthritis; and inhibition or prevention of tumor metastasis. See Johnson et al., supra.

A number of competitive inhibitors of bacterial and mammalian collagenases are known. See Johnson et al., supra; Vencill et al., Biochemistry 24, 3149 (1985); Yiotakis et al., Eur. J. Biochem. 172, 761 (1988); Galardy and Grobelny, Biochemistry 22, 4556 (1983); Grobelny and Galardy, Biochemistry 24, 6145 (1985); Mookhtiar et al. (II), Biochemistry 27, 4299 (1988); Gray et al., Biochem. Biophys. Res. Comm. 101, 1251 (1981); Clark et al., Life Sciences 37, 575 (1985); Wallace et al., Biochem. J. 239, 797 (1986); and Mookhtiar et al. (I), Biochemistry 26, 1962 (1987).

Zn^{+2}-metallopeptidase inhibitors, including those of the present invention, to be described in detail below, may also find antibacterial application against bacteria whose

pathogenicity depends at least in part on Zn^{+2}-metallopeptidases produced by the bacteria.

Information about Zn^{+2} metallopeptidases gained from a variety of different types of studies has provided a basis for the design of inhibitors of the enzymes. Thus, numerous chemical and kinetic studies of synthetic substrates and inhibitors of the various enzymes have led to suppositions about the three-dimensional structures that an inhibitor would need to have for a good fit in the active site of an enzyme and about the chemical interactions involved in catalysis of proteolysis by an enzyme or inhibition of such catalysis. The availability of high-resolution molecular structures from X-ray diffraction studies of crystallized the Zn^{+2} metallopeptidases carboxypeptidase A and thermolysin, coupled with evidence that the Zn^{+2}-containing active sites of Zn^{+2}-metallopeptidases are similar in molecular structure and function chemically in similar ways in their catalytic activities, has provided additional information to guide the design of inhibitors that have appropriate structural and chemical properties to be inhibitors that are specific for one or a few of the types of Zn^{+2} metalloendopeptidases. The availability of amino acid sequences for metalloendopeptidases, including carboxypeptidases A, thermolysins from various sources, enkephalinases from various sources, and ACE's from various sources, has provided additional information suggestive of structures of active sites and binding sites for substrates and inhibitors and the structural requirements and chemical attributes of desirable inhibitors.

Still, the art of predicting compounds that will be effective as inhibitors of a particular Zn^{+2} metalloendopeptidase (i.e., enkephalinase, ACE, or collagenase) and designing inhibitors based on such predictions remains uncertain. (To be regarded as effective as an inhibitor, in the case of a competitive inhibitor, a compound should have a K_i of less than about 50 μM.) Experience has shown

that, notwithstanding information that might be available on an enzyme from studies of its molecular structure, its primary sequence, and the physical and chemical properties of its substrates and inhibitors, there remain numerous ill-understood factors that affect whether a particular compound will be an effective inhibitor. Predicting compounds that will be specific inhibitors for a particular type of Zn^{+2} metalloendopeptidase (i.e., enkephalinase or ACE or collagenase, with, in the case of a competitive inhibitor, an inhibition constant, K_i, for one type that is at least about two orders of magnitude lower than that for the other types) is even more uncertain, because often subtle, ill-understood differences among the enzymes are important in such predictions. Still more uncertain is the design of so-called "mechanism-based" inhibitors or enzymes, including Zn^{+2} metalloendo-peptidases, as such inhibitors must not only, like competitive inhibitors, physically occupy the active site of an enzyme to block access thereto of substrate but also be positioned with sufficient precision and stability in the active site to undergo chemical reaction(s) there to unmask reactivity of a functional group so that the activated functional group, in turn, can form a covalent bond with a moiety of the enzyme, usually in or near the active site. The task of designing inhibitors for an enzyme is further complicated when, as with enkephalinase, ACE and collagenase, the three-dimensional structure of the enzyme to atomic resolution (as from X-ray crystallographic studies), which can reveal many of the details pertinent for rational design of inhibitors of the enzyme, is not available to guide the design.

The known inhibitors for enkephalinase, ACE and collagenases are competitive inhibitors. As such, the inhibitors are only transiently held, non-covalently, in the enzyme's active site and are effective in blocking peptidase activity on natural substrates only during the time that they occupy the active site of the enzyme in a

way that blocks access thereto in a reactive orientation of such a substrate. Dissociation of the enzyme-inhibitor complex frees the enzyme to act upon its natural substrate. Undesirably, as competitive enzyme inhibitors are degraded or otherwise cleared from the body, the activity of the enzyme intended to be inhibited is quickly and substantially fully restored, because no enzyme is irreversibly inactivated by competitive inhibitors. Nonetheless, it would be desirable to have additional competitive inhibitors for enkephalinase, ACE and collagenases, particularly ones that have low inhibition constants (below about 1 Nm) for at least one of the enzymes of a species (especially human) or that are specific for one of the three types of enzymes (i.e., an inhibitor that has an inhibition constant for one of the types of enzyme that is in the nanomolar range and an inhibition constant for the other types of enzyme of the same species that is at least about 100 to 1000 times greater).

It would also be desirable to have irreversible inhibitors of enkephalinase, ACE and collagenases, which, by permanently inactivating the enzymes, would provide longer-lived inhibition thereof. In particular, mechanism-based inhibitors for the enzymes would be especially desirable. A mechanism-based inhibitor, otherwise sometimes referred to as a "suicide inhibitor," is capable, once it has formed a Michaelis complex through non-covalent interactions in the active site of the enzyme to be inhibited, of chemically interacting with moieties of the enzyme in the active site in a manner which enables a "latent" functional group of the inhibitor to be activated (sometimes referred to as "unmasked") so that the inhibitor then reacts, and forms covalent bonds, with residue(s) of the enzyme in, or very close to, the active site. If, in an encounter of the inhibitor with an active site of the enzyme to be inhibited, stable covalent bonds with the enzyme are formed, the enzyme will be irreversibly inhibited, because the active site will be permanently

occupied or blocked by the inhibitor. See, e.g., Walsh, Ann. Rev. Biochem. 53, 493 - 535 (1984); Walsh, Tetrahedron Lett. 38, 871 - 908 (1982).

Particularly preferred would be a mechanism-based inhibitor which would irreversibly inactivate only one type of Zn^{+2} metallopeptidase (e.g., only enkephalinase, or only ACE, or only collagenase) of a mammalian, and particularly the human, species.

The regioisomers, (R)-2-benzyl-5-cyano-4-oxopentanoic acid and (R)-3-benzyl-5-cyano- 4-oxopentanoic acid are mechanism based inhibitors of the zinc exopeptidase carboxypeptidase A (CPA). The α-cyano ketone group of these pentanoic acid derivatives is a latent functionality which is capable of being unmasked in the CPA active site to a reactive α-keto-ketenimine intermediate and, through the reactive intermediate, covalently bonding to a reactive amino acid side-chain in the active site. This modification of the active site irreversibly inactivates CPA.

The two pentanoic acid derivatives, which are effective mechanism-based inhibitors of CPA, are ineffective for inhibiting enkephalinase, ACE or collagenases, having competitive inhibition constants much greater than 50 μM for the enzymes and not being active as irreversible inhibitors of the enzymes. Even though the active sites of CPA, enkephalinase, ACE and collagenases are thought to be closely related in terms of the chemistry of their peptide bond cleavage mechanisms, it is apparent, from the results with the pentanoic acid derivatives, that there are significant differences between CPA, on the one hand, and enkephalinase, ACE and collagenases on the other.

Prior to the present invention, attempts to prepare 2-benzyl-5-cyano-4-oxopentanoic acid substantially free (i.e., contaminated with less than about 10 mole %) of its regioisomer, 3-benzyl-5-cyano-4-oxopentanoic acid, had been unsuccessful.

SUMMARY OF THE INVENTION

The invention entails potent inhibitors of enkephalinase, ACE and collagenases. The inhibitors of the invention are dipeptide-analog derivatives of 2-benzyl-5-cyano-4-oxopentanoic acid. The inhibitors of the invention are compounds of Formula I

$$X_1-(CR_3R_4)_z-(CR_5R_6)_x-CHR_9-(C=O)-X_2$$

I,

wherein X_1 is a functional group from which a Zn^{+2} metalloendopeptidase, at its active site, is capable of abstracting a proton to yield an activated functional group capable of forming a stable, covalent bond with a residue in the active site; z is 0 or 1, wherein, when z is 0, the group $-(CR_3R_4)_z-(CR_5R_6)_x-$ is not in the compound and the group X_1- is bonded directly to the group $-(CHR_9)-$; x is 0 if z is 0 or is 0 or 1 if z is 1, wherein, when x is 0 and z is 1, the group $-(CR_5R_6)_x-$ is not in the compound and the group $-(CR_3R_4)-$ is bonded directly to the group $-(CHR_9)-$; R_3, R_4, R_5, and R_6 are independently hydrogen or alkyl of 1 to 3 carbon atoms; R_9 is benzyl, alkyl of 1 to 5 carbon atoms, or hydrogen; X_2 is joined to the -CO- in an amide linkage and is selected from the group consisting of glycine, N-methyl-glycine, N-benzyl-glycine, D-alanine, L-alanine, β-alanine, D-phenylalanine, L-phenylalanine, D-leucine, L-leucine, 3-amino propionic acid, D-proline, L-proline, and the group X_3-X_4, wherein X_3 is joined to the $-CHR_9-CO-$ group in an amide linkage and is selected from the group consisting of L-proline, L-alanine, L-valine, L-leucine, and L-O-methyltyrosine; when X_3 is L-proline or L-alanine, X_4 is selected from the group consisting of L-arginine, L-proline, L-leucine, L-alanine, L-hydroxyproline, and L-homoarginine; and when X_3 is L-valine, L-leucine, or L-O-methyltyrosine, X_4 is selected from the group consisting of glycine, L-alanine, and the alkyl esters of glycine and alanine, wherein the alkyl is of 1 - 5 carbons; provided that, if R_9 is benzyl, x is 0; if X_2 is X_3-X_4 and X_3 is L-

proline or L-alanine, z is 1, x is 0, and R_9 is hydrogen or methyl; and if X_2 is X_3-X_4 and X_3 is L-leucine, L-valine or L-O-methyltyrosine, z is 1, x is 0 and R_9 is alkyl of 3 - 5 carbon atoms; and physiologically acceptable salts thereof. It is intended that all stereoisomers be included in the compounds of Formula I.

Among the groups X_1 are a group of formula $(N{\equiv}C)(CHR_{10})(C{=}O)-$, wherein R_{10} is hydrogen or alkyl of 1 to 3 carbons and with which isomerization can occur by proton abstraction, with transfer of a proton from the α-carbon, to give rise to a reactive ketoketenimine; a group of formula $X_5(CR_{11}R_{12})(CHR_{13})(C{=}O)-$, wherein X_5 is a good leaving group, such as fluoro, chloro or bromo, R_{11}, R_{12} and R_{13} are independently selected from hydrogen or alkyl of 1 to 3 carbon atoms, and with which proton abstraction can occur in an elimination reaction, with loss of a hydrogen from the carbon alpha to the carbonyl group and the X_5 group from the carbon beta to the carbonyl group, leading to formation of an α, β-unsaturated ketone, a reactive Michael acceptor; and a group of formula $HC{\equiv}C(CHR_{14})(C{=}O)-$, wherein R_{14} is hydrogen or alkyl of 1 to 3 carbon atoms, with which proton abstraction can lead to an allenic ketone, also a reactive Michael acceptor.

The inhibitors of the invention are useful as analgesics (i.e., the enkephalinase inhibitors) or antihypertensives (i.e., the ACE inhibitors or the enkephalinase inhibitors); and in antibacterial or therapeutic applications involving collagenase inhibition. The invention encompasses methods of treating pain or hypertension in mammals, including humans, suffering therefrom by administering to such a mammal an effective amount of an analgesic or antihypertensive, respectively, according to the invention.

The invention further encompasses 2-benzyl-5-cyano-4-oxopentanoic acid substantially free (i.e., contaminated with less than about 10 mole %) of its regioisomer, 3-benzyl-5-cyano-4-oxopentanoic acid, methods

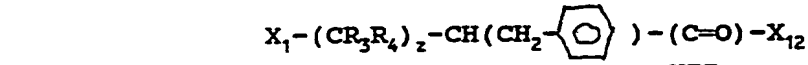
of making 2-benzyl-5-cyano-4-oxopentanoic acid substantially free of its regioisomer, 3-benzyl-5-cyano-4-oxopentanoic acid, and methods of making inhibitors according to the invention (wherein, with reference to Formula I, X_1 is $(N\equiv C)(CH_2)(C=O)-$, x is 0, z is 1, R_3 and R_4 are both hydrogen and R_9 is benzyl) using as a starting material 2-benzyl-5-cyano-4-oxopentanoic acid substantially free of its regioisomer, 3-benzyl-5-cyano-4-oxopentanoic acid.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compounds of Formula I, described supra.

The discovery which underlies the present invention is that the presence of a functional group X_1 in a compound which is capable of binding in the active site of a Zn^{+2} metalloendopeptidase provides a mechanism-based inhibitor for the enzyme.

In addition to the compounds of the invention, the invention provides a method of reducing pain in a mammal suffering therefrom comprising administering to said mammal a pain-reducing-effective amount of a compound of Formula XII:



XII,

wherein z is 0 or 1, wherein, if z is 0, the group $-(CR_3R_4)_z-$ is not present in the compound and X_1 is bonded directly to the group $-CH(CH_2-\langle\bigcirc\rangle)-$; R_3, and R_4 are independently hydrogen or alkyl of 1 - 3 carbon atoms; and X_{12} is joined to the $-(C=O)-$ in an amide linkage and is selected from the group consisting of glycine, N-benzyl-glycine, L-alanine, D-alanine, L-phenylalanine, D-phenylalanine, L-leucine, D-leucine, and 3-amino propionic acid; or a pharmacologically acceptable salt thereof.

Still further, the invention provides a method for treating hypertension in a mammal suffering therefrom comprising administering to said mammal an antihypertensive-effective amount of a compound of Formula I, wherein X_2 is other than X_3X_4; or a physiologically acceptable salt thereof.

Still further, the invention provides a method for inhibiting collagenase comprising combining with a collagenase a collagenase-inhibiting effective amount of a compound of Formula I, wherein X_2 is X_3X_4; or a physiologically acceptable salt thereof. Therapeutic applications of collagenase inhibition are cited supra.

In another aspect, the invention provides 2-benzyl-5-cyano-4-oxopentanoic acid substantially free of 3-benzyl-5-cyano-4-oxopentanoic acid.

In still another aspect, the invention provides a method of making (R)-2-benzyl-5-cyano-4-oxo-pentanoic acid, substantially free of 3-benzyl-5-cyano-4-oxo-pentanoic acid, which method comprises the steps of:

(a) reacting (R,S)-benzylsuccinic acid with methanolic Hcl to make (R,S)-dimethyl-α-benzyl succinate;

(b) treating the product of step (a) with α-chymotrypsin until substantially all of the (R) enantiomer of said product of step (a) is converted to (R)-2-benzyl-3-carbomethoxy-propionic acid or the conjugate base thereof;

(c) acidifying the product of step (b) to convert substantially all of said product to the acid form;

(d) reducing the product of step (c) to make methyl-(R)-3-benzyl-4-hydroxybutanoate;

(e) reacting said methyl-(R)-3-benzyl-4- hydroxy butanoate with $Li(CH_2CN)$ to yield (R)-4-benzyl-2-cyanomethyl-(R,S)-2- hydroxy-tetrahydrofuran; and

f) oxidizing said (R)-4-benzyl-2-cyanomethyl-(R,S)-2-hydroxytetrahydrofuran to yield (R)-2-benzyl- 5-cyano-4-oxo-pentanoic acid.

Reference herein to a compound or a formula for a compound is, unless otherwise qualified, to all stereoisomers of the compound. The designation of "R" or "S" as the configuration at an asymmetric carbon of a compound is based on Cahn-Ingold-Prelog convention rules. Reference to an amino acid, unless the configuration at its asymmetric carbon is specified otherwise, is to the L-enantiomer. Three letter abbreviations used for amino acids are the standard three letter abbreviations used in the art, including "Har" for L-homoarginine and "Hyp" for 4-hydroxy-L-proline.

The compounds of Formula I of the present invention are inhibitors of enkephalinase or angiotensin-converting enzyme ("ACE") or both of these enzymes from mammals, including humans, or inhibitors of bacterial or mammalian collagenases.

The amino acid sequences of the human, rat and rabbit enkephalinases have been deduced from cDNAS for the enzymes. Malfroy et al., Biochem Biophys. Res. Comm. 144, 59 - 66 (1987); Devault et al., EMBO J. 6, 1317 - 1322 (1987); Malfroy et al., FEBS Lett. 229, 206 - 210 (1988). The amino acid sequence of human ACE is provided by Soubrier et al., Proc. Natl. Acad. Sci. (USA) 85, 9386 (1988).

Methods for preparing physiologically acceptable salts of the compounds of Formula I, which are weak acids, are well known. Among such salts are the sodium, potassium, ammonium, magnesium, and calcium salts.

Especially preferred among the endopeptidase inhibitors of the invention, of Formula I, are those wherein X_1 is $(N{\equiv}C)(CH_2)(C{=}O)-$ or $Cl(CH_2)_2(C{=}O)-$ and all of R_3, R_4, R_5, and R_6, if present in the compound, are hydrogen and which are mechanism-based inhibitors of enkephalinase, ACE or a collagenase on account of an activated intermediate, with an "unmasked," reactive ketoketenimine or α,β-unsaturated ketone group, which is produced in the active

site of the enzyme from the $(N\equiv C)(CH_2)(C=O)-$ group or the $Cl(CH_2)_2(C=O)-$, respectively.

The compounds of Formula I, which are mechanism-based inhibitors, are also necessarily substrates of the enzyme. In some encounters between such a compound of the invention and enkephalinase, ACE, or a collagenase, the compound will be changed in a reaction catalyzed by the enzyme and will diffuse away from the active site of the enzyme before a covalent bond with a moiety in the active site can be formed by this changed inhibitor compound. In other encounters, after such a reaction is catalyzed, a subsequent reaction with a moiety in the active site of the enzyme will occur with the changed inhibitor to effect a covalent linkage between the inhibitor and the enzyme and irreversibly inactivate the enzyme. The "partition ratio" of inactivation of an enzyme by a mechanism-based enzyme inhibitor with an enzyme is defined as the negative of the time derivative of the concentration of the inhibitor divided by the time derivative of the concentration of inactivated enzyme. The partition ratio is one less than the average number of molecules of inhibitor with which the enzyme must catalyze formation of an activated intermediate before the intermediate will react with and inactivate the enzyme. Measurement of the partition ratio of inactivation of an enzyme by a mechanism-based inhibitor is readily carried out by the skilled. A partition ratio for inactivation of an enzyme by a mechanism-based inhibitor of 5000 or less is desirable; especially preferred are partition ratios of 2000 or less.

A measure of the specificity of a mechanism-based inhibitor for one of a set of enzymes is provided by the partition ratios for inactivation of the various enzymes by the inhibitor; if the partition ratio for one of the enzymes is very much lower than those for the other enzymes, the mechanism-based inhibitor can be said to be specific in mechanism-based inhibition for the one enzyme of the set.

The effectiveness of the enkephalinase inhibitors, ACE inhibitors and collagenase inhibitors of the invention as antinociceptive, antihypertensive and collagenase inhibiting agents, respectively, is ascertained by their ability to inhibit purified enkephalinase, ACE or collagenase, respectively, in vitro.

The compounds listed in Tables 1, 2, 3 and 4 are particularly preferred enkephalinase, ACE and collagenase inhibitors, respectively. It will be noted that several compounds are both ACE inhibitors and enkephalinase inhibitors.

TABLE 1

ENKEPHALINASE INHIBITORS*

$NCCH_2-CO-(CH_2)_i-CH(CH_2C_6H_5)-CO-L$-glycine

$NCCH_2-CO-(CH_2)_i-CH(CH_2C_6H_5)-CO-L$-alanine

$NCCH_2-CO-(CH_2)_i-CH(CH_2C_6H_5)-CO-D$-alanine

$NCCH_2-CO-(CH_2)_i-CH(CH_2C_6H_5)-CO-NH-CH_2-CH_2-COOH$

$NCCH_2-CO-(CH_2)_i-CH(CH_2C_6H_5)-CO-L$-leucine

$NCCH_2-CO-(CH_2)_i-CH(CH_2C_6H_5)-CO-L$-phenylalanine

(i is 0 or 1, wherein, when i is 0, the CH_2 group is not present in the compound and the α-cyano ketone group is bonded directly to the carbon to which the benzyl group is bonded.)

*Preferred configuration at the asymmetric center outside the amino acid moiety is R.

TABLE 2

ACE INHIBITORS*

$NCCH_2-CO-CH_2-CH(CH_3)-CO-L-proline$

$NCCH_2-CO-CH_2-CH_2-CH(CH_3)-CO-L-proline$

$NCCH_2-CO-CH_2-CH_2-CO-L-proline$

$NCCH_2-CO-CH_2-CH_2-CH_2-CO-L-proline$

$NCCH_2-CO-CH_2-CH(CH_2C_6H_5)-CO-L-proline$

$NCCH_2-CO-CH_2-CH(CH_2C_6H_5)-CO-L-alanine$

$NCCH_2-CO-CH_2-CH(CH_2C_6H_5)-CO-D-alanine$

$NCCH_2-CO-CH_2-CH(CH_2C_6H_5)-CO-L-phenylalanine$

$NCCH_2-CO-CH_2-CH(CH_2C_6H_5)-CO-L-leucine$

$NCCH_2-CO-CH_2-CH(CH_2C_6H_5)-CO-L-glycine$

$NCCH_2-CO-CH_2-CH(CH_2C_6H_5)-CO-N(CH_3)-CH_2-COOH$

*Preferred configuration at the asymmetric center outside the amino acid moiety is R.

TABLE 3

BACTERIAL COLLAGENASE INHIBITORS

$NCCH_2-CO-CH_2-CH_2-CO-L-proline-L-arginine$

$NCCH_2-CO-CH_2-CH_2-CO-L-proline-L-proline$

$NCCH_2-CO-CH_2-CH_2-CO-L-proline-L-leucine$

$NCCH_2-CO-CH_2-CH_2-CO-L-proline-L-alanine$

$NCCH_2-CO-CH_2-CH_2-CO-L-proline-L-hydroxyproline$

$NCCH_2-CO-CH_2-CH_2-CO-L-proline-L-homoarginine$

$NCCH_2-CO-CH_2-CH_2-CO-L-alanine-L-arginine$

$NCCH_2-CO-CH_2-CH_2-CO-L-alanine-L-proline$

$NCCH_2-CO-CH_2-CH_2-CO-L-alanine-L-leucine$

$NCCH_2-CO-CH_2-CH_2-CO-L-alanine-L-alanine$

$NCCH_2-CO-CH_2-CH_2-CO-L-alanine-L-hydroxyproline$

$NCCH_2-CO-CH_2-CH_2-CO-L-alanine-L-homoarginine$

TABLE 4

MAMMALIAN COLLAGENASE INHIBITORS

$NCCH_2$-CO-CH_2-CH(^{i}Bu)-CO-L-leucine-glycine ethyl ester

$NCCH_2$-CO-CH_2-CH(^{i}Bu)-CO-L-leucine-L-alanine ethyl ester

$NCCH_2$-CO-CH_2-CH(^{i}Bu)-CO-L-O-methyltyrosine-glycine ethyl ester

$NCCH_2$-CO-CH_2-CH(^{i}Bu)-CO-L-O-methyltyrosine-L-alanine ethyl ester

$NCCH_2$-CO-CH_2-CH(^{i}Bu)-CO-L-valine-glycine ethyl ester

^{i}Bu = isobutyl.

Methods of assaying enkephalinase inhibitors, ACE inhibitors, and collagenase inhibitors for capacity to inhibit the respective enzymes, including methods of purifying enzymes for use in the assay methods, are known to those skilled in the study of the enzymes. Methods for enkephalinase inhibitors, ACE inhibitors and bacterial collagenase inhibitors are described in the Examples. For mammalian collagenase inhibitors, see Johnson et al., supra, at pages 4 - 5, and the references cited there.

It is contemplated that the inhibitors of the invention will be administered under the guidance of a physician or veterinarian to relieve pain in a human or other mammal suffering therefrom (in the case of the enkephalinase inhibitors of the invention) or to reduce blood pressure in a human or other mammal suffering from hypertension (in the case of the ACE inhibitors or enkephalinase inhibitors of the invention), or for both purposes in the case of inhibitors of the invention which are effective as inhibitors of both enkephalinase and ACE, or for any of a number of therapeutic applications, such as treatment of corneal ulcers or periodontal disease, in the case of inhibitors of the invention which are effective as inhibitors of collagenases.

With respect to the enkephalinase or ACE inhibitors, administration will be parenterally, preferably intravenously, in unit doses or by continuous infusion, of an inhibitor or a physiologically acceptable salt thereof dissolved in any physiologically acceptable diluent, such

as physiological saline, phosphate buffered saline, or the like. The route of administration (e.g., intravenous, intramuscular, intraperitoneal, subcutaneous), mode of administration (e.g., by unit doses or continuous infusion), and dosage regimen will vary somewhat depending on the inhibitor employed, the species, age, weight and general medical condition of the mammal being treated, and the particular condition of the mammal for which the inhibitor is being administered. Determining these factors for a particular mammal being treated for a particular condition with a particular inhibitor will be routine for the pharmacologist, physician or veterinarian of ordinary skill. Generally, in the case of treating humans with an inhibitor according to the invention, a dose of inhibitor or physiologically acceptable salt thereof of between about 0.01 mg/kg body weight per day and 100 mg/kg body weight per day, infused continuously, administered in several equal doses per day, or administered in a single dose per day, will be effective to relieve pain (in the case of enkephalinase inhibitors) or reduce hypertension (in the case of ACE inhibitors or enkephalinase inhibitors).

With respect to the collagenase inhibitors according to the invention, administration may be topical in a suitable, physiologically acceptable vehicle (e.g., cream, solution) for application to the eye, in the case of use for treatment of corneal ulceration, or application into the gingival crevice or subgingival space, in the case of use for treatment of periodontal disease. The collagenase inhibitors may also be administered parenterally, in unit doses or by continuous infusion, at or near the site on the body of the mammal being treated at which inhibition of collagen degradation is desired. The inhibitor or a physiologically acceptable salt thereof will, for administration, be dissolved in any physiologically acceptable diluent, such as physiological saline, phosphate buffered saline, or the like. The route of administration, mode of administration (e.g., by unit doses or continuous

infusion), and dosage regimen will vary somewhat depending on the inhibitor employed, the species, age, weight and general medical condition of the mammal being treated, and the particular condition of the mammal for which the inhibitor is being administered. Determining these factors for a particular mammal being treated for a particular condition with a particular inhibitor will be routine for the pharmacologist, physician or veterinarian of ordinary skill. Generally, in the case of treating humans with a collagenase inhibitor according to the invention, a dose of inhibitor or physiologically acceptable salt thereof of between about 0.1 mg per day and 100 mg per day, infused continuously, or administered by any route, including topically or by injection into or near the site at which collagenase inhibition is desired, in several equal doses per day, or a single dose per day, will be effective to achieve the desired inhibition of collagen degradation. In certain applications, such as treating periodontal disease, inhibitors according to the invention of bacterial collagenases and mammalian collagenases may be employed in combination.

The synthesis of an inhibitor of the invention, wherein, with reference to Formula I, X_1 is $NC(CH_2)(C=O)-$, employs an analog of 5-cyano-4-oxo-pentanoic acid, 6-cyano-5-oxo-hexanoic acid, or 2-benzyl-4-cyano-3-oxo-butanoic acid as starting material.

Preparation of (R)-2-benzyl-5-cyano-4-oxo-pentanoic acid is described presently. The preparation of 2-benzyl-5-cyano-4-oxo-pentanoic acid substantially free of its 3-benzyl regioisomer had not, until the present invention, been achieved; the method of preparation presently described is thus most advantageous for preparation of the compounds of Formula I wherein R_9 is benzyl and X_1 is $NC(CH_2)(C=O)-$, because it makes possible the synthesis of these compounds substantially free of contamination by the analogs based on the 3-benzyl regioisomer.

The synthesis of 2-benzyl-5-cyano-4-oxo-pentanoic acid substantially free of its 3-benzyl regioisomer begins by refluxing (R,S)-benzylsuccinic acid in methanolic Hcl to yield a racemic mixture of the dimethyl ester of succinic acid. This racemic mixture is then subjected to esterolysis with α-chymotrypsin, which yields the optically pure (R)-ester acid. Acidification yields (R)-2-benzyl-3-carbomethoxy propionic acid.

Subsequent treatment of (R)-2-benzyl- 3-carbomethoxy-propionic acid in THF with BH_3·THF yields (R)-methyl-3-benzyl-4-hydroxybutanoate.

Next, acetonitrile and n-BuLi are reacted to form the lithium salt of the anion of acetonitrile, $Li^+(CH_2CN)^-$, to which is added (R)-methyl-3-benzyl- 4-hydroxybutanoate to yield (R)-4-benzyl-2-cyanomethyl-(R,S)-2-hydroxytetra-hydrofuran.

Then, (R)-4-benzyl-2-cyanomethyl-(R,S)-2-hydroxytetra-hydrofuran is subjected to Jones oxidation by adding said solution slowly to a solution of acetic, chromic, and sulfuric acids to give (R)-2-benzyl-5-cyano-4-oxo-pentanoic acid.

(R)-2-benzyl-5-cyano-4-oxo-pentanoic acid is employed in a mixed anhydride method to make the N-substituted inhibitors of the invention. N-methyl morpholine and isobutylchloroformate are sequentially added to (R)-2-benzyl-5-cyano-4-oxo-pentanoic acid, the reaction mixture diluted, and the amino acid methyl ester corresponding to the desired amino acid constituent of the inhibitor is added. Alkaline hydrolysis in aqueous methanol of the methyl ester derivative then provides an inhibitor of the invention.

The synthesis of 2-benzyl-3-oxo-4-cyano-butanoic acid begins with the partial hydrolysis of the dimethyl ester of 2-benzylmalonic acid with NaOH in methanol to give the half ester of 2-benzylmalonic acid. Treatment of the compound with oxalyl chloride in the presence of a catalytic amount of dimethylformamide provides the acid chloride, which is

reacted with the sodium anion of t-butyldimethylcyano-acetate. An acid quench gives methyl 2-benzyl-3-oxo-4-cyano-butanoate. Alkaline hydrolysis of the methyl ester in aqueous methanol provides 2-benzyl-3-oxo-4-cyano-butanoic acid. The compound can be used to make inhibitors according to the invention following the same strategy used for the homologue, (R)-2-benzyl-4-oxo-5-cyano-pentanoic acid. The diastereomeric dipeptide analogue inhibitors are not separated by reverse phase chromatography due to epimerization from ketoenol exchange at the asymmetric carbon of the malonyl moiety.

It will be clear to those of ordinary skill that 2-alkyl-5-cyano-4-oxo-pentanoic acids and 5-cyano-4-oxo-pentanoic acid may be synthesized from the corresponding 3-alkyl-3-carbomethoxy propionic acid and the commercially available succinic acid monomethylester chloride, respectively, following the strategy outlined for 2-benzyl-4-cyano-3-oxo-butanoic acid. The synthesis of 3-methyl-3-carbomethoxy propionic acid is known in the art. Cushman et al. Biochemistry 16, 5484 (1977). 3-iso-butyl-3-carbomethoxy propionic acid is prepared by Arndt-Eistert homologation of the commercially available monomethyl ester of 2-iso-butyl-malonic acid.

2-alkyl-5-cyano-4-oxo-pentanoic acid and 5-cyano-4-oxo-pentanoic acid are employed as described above to make inhibitors according to the invention.

It will also be clear to the skilled that the inhibitors of the invention, which are derivatives based on 2-alkyl-6-cyano-5-oxo-hexanoic acid and 6-cyano-5-oxo-hexanoic acid may be synthesized by starting from 4-alkyl-4-carbomethoxy-butanoic acid, or glutaric acid monomethylester chloride, respectively, and following the strategy outlined for 2-benzyl-4-cyano-3-oxo-butanoic acid.

A 4-alkyl-4-carbomethoxy-butanoic acid is conveniently obtained by homologation of the corresponding 3-alkyl-3-carbomethoxy propionic acid using the Arndt-Eistert reaction. Thus, reaction of the latter compound with

oxalyl chloride in the presence of a catalytic amount of dimethylformamide followed by treatment of the resulting acid chloride with etherial diazomethane provides the diazolactone derivative. Exposure to Ag_2O and then acidic treatment affords the 4-alkyl-4-carbomethoxy-butanoic acid. Alternatively, the method of Cushman et al., supra, can be used employing fractional crystallization of the dicyclohexylammonium salts of the monomethyl esters.

When desired, diastereomers can be separated employing reverse phase chromatography.

The following Examples are included to aid in the understanding of the invention. They are not intended to limit the scope of the invention as defined by the appended claims.

EXAMPLE 1

(R,S)-dimethyl-α-benzyl succinate

To 100 ml of anhydrous methanol at 0°C and under N_2 was added 4.5 ml (65 mmoles) of acetyl chloride over a 5-minute period. The solution was stirred at 0°C for 15 minutes and then warmed to room temperature. (R,S)-benzylsuccinic acid (6 grams, 28.8 mmoles) was then added, and the solution was refluxed for 3 hours. After removal of the methanol under vacuum, the residual oil was taken up in ethyl acetate (200 ml) and washed with saturated $NaHCO_3$ and brine, and then dried over anhydrous $MgSO_4$. Concentration of the solution afforded the dimethyl ester as an oil (6.5 grams, 96%), which was pure by NMR. ^1H NMR ($CDCl_3$) delta 7.31-7.14 (m, 5H), 3.67 (s, 3H), 3.64 (s, 3H), 3.15-3.02 (m, 2H), 2.80-2.64 (m, 2H), 2.41 (dd, J = 16.8, 4.9 Hz, 1H); IR (neat) 3029, 2953, 1731 cm^{-1}. High- resolution mass spectrum calculated for $(M+H)^+$: 237.1127; found: 237.1122.

(R)-2-benzyl-3-carbomethoxy-propionic acid

The procedure of Cohen, S.G. and Milovanovic, A., (1968) J. Am. Chem. Soc. 90:3495-3502] for the esterolysis of (R,S)-α-benzyl succinate.

To a suspension of (R,S)-dimethyl-α-benzyl succinate (6.55 grams, 28 nmoles) in 100 ml of H_2O at 23°C was added a solution of 800 mg α-chymotrypsin (EC 3.4.21.1) in 60 ml of H_2O. A constant pH of 7.2 was maintained by titrating the reaction mixture with 0.1 N NaOH using a pH-Stat. After 18 hours, 140 ml of the NaOH solution was consumed, indicating 50% hydrolysis of the racemic substrate. After extraction of the suspension with ether, the aqueous phase was acidified, to pH 2.00, and then concentrated in vacuo. The residue was sonicated with 300 ml of ether, filtered, washed with brine, and dried over anhydrous $MgSO_4$. Concentration of the organic extract provided 3.05 grams of the optically active monomethyl ester- acid product (98%), which was pure by NMR. ^1H NMR ($CDCl_3$) delta 7.33-7.05 (m, 5H), 3.65 (s, 3H), 3.16 (m, 2H), 2.78 (m, 1H), 2.66 (dd, J = 17.9 Hz, 1H), 2.41 (dd, J = 4.4, 17 Hz, 1H); IR (neat) 3500-3300 (br), 1738, 1713 cm^{-1}. High-resolution mass spectrum calculated for $(M+H)^+$: 223.0970; found 223.0966. $[\alpha]_D^{25}$ = +15.5 (c 1.1, ethyl acetate).

(R)-methyl 3-benzyl-4-hydroxybutanoate

To a stirred solution of (R)-2-benzyl-3- carbomethoxy-propionic acid (1.695 g, 7.6 mmol) in THF (10 ml) at 0°C under N_2 was added BH_3·THF (10 ml of a 1.0 M solution in THF, 10 mmol) dropwise. After stirring for 15 minutes at 0°C, the flask was placed in the freezer for 16 hours. The reaction was quenched at 0°C with MeOH (5 ml), stirred for 30 minutes at 0°C, and then stirred for 30 minutes at room temperature (RT). The solution was diluted with MeOH (10 ml), and the solvent was removed. This procedure was repeated twice. The residue was dissolved in Et_2O (100 ml), washed with saturated $NaHCO_3$ (10 ml), brine (10 ml), and dried over $MgSO_4$. Removal of the solvent in vacuo gave 1.36

g (86%) of the product as an oil. ^{1}H NMR (350 Mhz, $CDCl_3$) delta 7.32-7.16 (5H, m), 3.64 (3H, s), 3.63-3.49 (2H, m), 2.75-2.70 (1H, dd, J = 6.6, 13.6 Hz), 2.62-2.56 (1H, dd, J = 6.7, 13.5 Hz), 2.46-2.33 (3H, m). IR (thin film) 3453 (b), 3026, 2950, 1732 cm^{-1}.

(R)-4-benzyl-2-cyanomethyl-(R,S)-2-hydroxytetrahydrofuran

A solution of n-BuLi (5.4 ml of a 2.5 M solution in hexane, 13.5 mmol) in THF (20 ml) was cooled to -78°C under N_2, and CH_3CN (0.64 ml, 12.3 mmol) in THF (10 ml) was added dropwise. A white precipitate formed after addition of the solution, and the reaction mixture was stirred for 1 hour at -78°C. (R)-methyl-3-benzyl- 4-hydroxybutanoate (0.64 g, 3.1 mmol) in THF (10 ml) was then added dropwise, and the reaction mixture was stirred for 1 hour at -78°C, followed by 1 hour at 0°C. The reaction was quenched with 5% Hcl (7 ml) and diluted with Et_2O (50 ml). The organic phase was separated, and the aqueous layer as extracted with Et_2O (20 ml). The ethereal extracts were combined, washed with brine, and then dried over $MgSO_4$. The solvent was removed, and the residue was flash-chromatographed using double elution and 1% i-prOH/30% EtOAc/hexane as eluent, to give 645 mg (97%) of the product as an oil. ^{1}H NMR (350 Mhz, $CDCl_3$) delta 7.32-7.14 (5H, m), 4.16-4.11 (0.7H, t, J = 8.0 Hz), 4.06-4.01 (0.3H, dd, J=7.3, 8.2 Hz), 3.83-3.78 (0.3H, t, J = 8.3 Hz), 3.70-3.65 (0.7H, t, J = 8.3 Hz), 2.93-2.69 (5H, m), 2.28-2.19 (1H, m), 1.95-1.89 (0.3H, dd, J = 7.1, 13.6 Hz), 1.79-1.72 (0.7H, dd, J = 10.0, 12.8 Hz). IR (thin film) 3418 (b), 2930, 2258 cm^{-1}.

(R)-2-benzyl-5-cyano-4-oxo-pentanoic acid

A solution of 4-benzyl-2-cyanomethyl-2-hydroxy-tetrahydrofuran (26 mg, 0.12 mmol) in acetic acid (1.2 ml) was added very slowly to a solution of $H_2Cr_2O_7$ (0.40 ml), H_2SO_4 (0.20 ml), H_2O (0.40 ml) and acetic acid (1.6 ml). After addition was complete, the solution was stirred for 1 hour. The reaction mixture was diluted with H_2O (10 ml) and

extracted with Et_2O 3x (2 ml each). The combined ethereal extracts were washed with brine (1 ml) and dried over $MgSO_4$. The solvent was removed, the residue was redissolved in toluene (5 ml), and the solution was concentrated in vacuo. Flash chromatography of the residue using 0.5% HOAc/35% EtOAc/ hexane as eluent afforded 14 mg product (50%) as an oil. ^1H NMR (360 Mhz, $CDCl_3$) delta 7.34-7.16 (5H, m), 3.37 (2H, bs), 3.32-3.17 (2H, m), 2.83-2.76 (2H, dd, J = 9.3, 13.5 Hz), 2.54-2.48 (1H, dd, J= 4.4, 17.4 Hz). IR (thin film) 3200 (b), 2916, 2264, 1731, 1713 cm^{-1}.

N-[(R)-2-benzyl-5-cyano-4-oxo-pentanoyl]-L-alanine methyl ester

To 50 mg (.216 mmole) of (R)-2-benzyl-5-cyano-4-oxo-pentanoic acid in 1 ml of $CHCl_3$ under N_2 was successively added 25 μl (.238 mmole) of N-methyl morpholine and 31 μl (.238 mmole of isobutylchloroformate). After stirring for 20 minutes at 23°C, 30 μl (.273 mmole) of N-methyl morpholine and 33 mg (.238 mmole) of L-alanine methyl ester·hydrochloride were added. The reaction mixture was stirred at 23°C for 20 hours. Subsequently, the reaction mixture was diluted with 25 ml of ether and washed with 5% citric acid, saturated $NaHCO_3$ and brine. The ethereal layer was dried over anhydrous $MgSO_4$ and concentrated in vacuo to provide 63 mg (92%) of the desired product as an oil. ^1H NMR ($CDCl_3$) delta 7.35-7.14 (m, 5H), 6.00 (bd, J = 7.2 Hz, 1H), 4.46 (m, 1H), 3.72 (s, 3H), 3.48 (m, 2H), 3.05 (m, 3H), 2.72 (m, 1H), 2.50 (m, 1H), 1.35 (d, J = 7.2 Hz, 3H). IR (thin film) 3314, 2952, 2261, 1731, 1660, 1651 cm^{-1}.

N-[(R)-2-benzyl-5-cyano-4-oxo-pentanoyl]-L-alanine

To a solution of 49 mg (0.15 mmole) of N-[(R)-2-benzyl-5-cyano-4-oxo-pentanoyl]-L-alanine methyl ester in 2 ml of CH_3OH was added 2 ml of H_2O, and the solution was cooled to 0°C under argon. To this mixture was added 0.31 ml of a 1 N NaOH solution (.62 mmole), and

the reaction mixture was stirred at 0°C for 1 hour, followed by 2 hours at 23°C. The mixture was diluted with 8 ml of brine and acidified to pH 2 with 10% Hcl. The aqueous layer was then extracted with ether (4 x 2 ml), and the combined ethereal layers were washed with brine, dried over anhydrous $MgSO_4$, and concentrated to give 43 mg (92%) of the product as an oil. ^1H NMR ($CDCl_3$) 7.43-7.34 (m, 5H), 6.25 (bd, 1H), 4.44 (m, 1H), 3.50 (m, 2H), 3.04 (m, 3H), 2.72 (m, 1H), 2.52 (bd, 1H), 1.39 (d, 3H). IR (thin film) 3332, 2941, 3028, 2265, 1731, 1714, 1693, 1682, 1660, 1651 cm^{-1}.

The dipeptide-analogs derivatized with the methyl ester of L-leucine, L-phenylalanine, or glycine were synthesized analogously to the method as described for the L-alanine methyl ester derivative.

N-[(R)-2-benzyl-5-cyano-4-oxo-pentanoyl]-glycine methyl ester

Yield = 67% ^1H NMR ($CDCl_3$) delta 7.37-7.15 (m, 5H), 4.65 (d, J = 17.5 Hz, 1H), 3.811 (m, 4H), 3.25 (dd, J = 13.8, 4.8 Hz, 1H), 3.14 (m, 1H), 2.75 (dd, J = 13.8, 9.1 Hz, 1H), 2.70 (d, J = 17 Hz, 1H), 2.55 (d, J = 17 Hz, 1H), 2.34 (dd, J = 8.7, 13.5 Hz, 1H), 2.02 (dd, J = 8.7, 13.5 Hz, 1H). IR (thin film) 3378, 2934, 2256, 1747, 1693, 1681 cm^{-1}.

N-[(R)-2-benzyl-5-cyano-4-oxo-pentanoyl]-L-phenylalanine methyl ester

Yield = 82%. ^1H NMR ($CDCl_3$) delta 7.34-7.07 (m, 10H), 5.96 (d, J = 7.8 Hz, 1H), 4.77 (m, 1H), 3.67 (s, 3H), 3.40 (s, 2H), 2.87 (m, 5H), 2.68 (m, 1H), 2.45 (m, 1H). IR (thin film) 3353, 3029, 2953, 1737, 1660.

N-[(R)-2-benzyl-5-cyano-4-oxo-pentanoyl]-L-leucine methyl ester

Yield = 79%. ^{1}H NMR ($CDCl_3$) delta 7.34-7.14 (m, 5H), 5.87 (bd, J = 8 Hz, 1H), 4.50 (m, 1H), 3.69 (s, 3H), 3.48 (m, 2H), 3.04 (m, 3H), 2.96 (m, 1H), 2.49 (m, 1H), 1.57 (m, 3H), .90 (m, 6H). IR (thin film) 3318, 2957, 2261, 1731, 1660, 1651 cm^{-1}.

The dipeptide-analogs derivatized with L-leucine, L-phenylalanine, and glycine were synthesized in the same manner as the L-alanine derivative by hydrolysis of their methyl esters.

N-[(R)-2-benzyl-5-cyano-4-oxo-pentanoyl]-L-leucine

Yield = 91%. ^{1}H NMR ($CDCl_3$) delta 7.4-7.12 (m, 5H), 6.19 (d, 1H), 4.50 (m, 1H), 3.49 (d, J = 8 Hz, 2H), 3.06 (m, 3H), 2.71 (m, 1H), 2.52 (m, 1H), 1.58 (m, 3H), .92 (m, 6H). IR (thin film) 3333, 2950, 2257, 1731, 1714, 1693, 1681, 1667, 1651 cm^{-1}.

N-[(R)-2-benzyl-5-cyano-4-oxo-pentanoyl]-glycine

Yield = 38%. ^{1}H NMR (CD_3OD) delta 7.35-7.17 (m, 5H), 4.03 (m, 1H), 3.31 (m, 2H). IR (thin film) 3356, 3028, 2931, 2258, 1731, 1713, 1693, 1681, 1651 cm^{-1}.

N-[(R)-2-benzyl-5-cyano-4-oxo-pentanoyl]-L-phenylalanine

Yield = 90%. ^{1}H NMR ($CDCl_3$) delta 7.37-7.08 (m, 10H), 6.16 (d, J = 7.6 Hz, 1H), 4.75 (m, 1H), 3.39 (s, 2H), 3.16 (dd, J = 9.7, 5.3 Hz, 1H), 2.99 (m, 4H), 2.66 (m, 1H), 2.46 (m, 1H). IR (thin film) 3330, 3028, 2937, 2262, 1731, 1714, 1693, 1681, 1667, 1651 cm^{-1}.

EXAMPLE 2

Enkepalinase from rabbit kidney cortex was purified by immunoaffinity chromatography using a monoclonal antibody. Biochem. Biophys. Res. Commun. 131, 255 (1985).

The assay for enkephalinase is carried out in vitro using the fluorescent substrate, dansyl-D-Ala-Gly-Phe-(p-NO_2)-Gly (referred to herein as "DAGNPG") in accordance with the procedure of Florentin et al., Anal Biochem. 141, 62 (1984). Fluorometric assays are performed at 37°C on a spectrofluorometer (e.g. Gilford Fluoro IV, Gilford Instruments Co.)equipped with a temperature-controlled cell holder.

The enkephalinase inactivator assay is carried out as follows: Enkephalinase (4 μg) and various amounts of inhibitor are mixed (final volume = 53μl) and inactivation is allowed to occur at 23°C for 5 hours. Reaction mixtures contain 0.15 M Tris acetate, 1% octylglucoside, 5% dioxane, pH 7.4, 3.8 μg enkephalinase (rabbit kidney) and varying amount of enkephalinase inhibitor. At various times, a 5 μl aliquot of the reaction mixture is withdrawn and added to an assay solution which contains 0.1 mM DAGNPG and 50 mM Tris hydrochloride, pH 7.4. Initial reaction rates (for the first 10% of the reaction) are monitored continuously by measuring the increase of fluorescence at an excitation wavelength of 342 nm and emission wavelength of 562 nm. A plot of the initial rate versus time gives a time course of inactivation of the enzyme.

EXAMPLE 3

Angiotensin converting enzyme from frozen rabbit lung is purified according to the procedure of Das and Soffer, J. Biol. Chem. 250, 6762 (1975).

The enzyme is assayed using hippuryl-L-histidyl-L-leucine according to the procedure of Cheung and Ondetti, Biochim. Biphys. Acta 293, 451 (1973), by following the release of histidyl-leucine in 100 mM potassium phosphate, 300 mM NaCl, pH 8.3 and a single 30 minute time point. The reaction is initiated by the addition of enzyme and terminated by addition of 0.3 M NaOH. The fluorometric assay of the released histidyl-leucine is then performed using o-pthaldialdehyde in methanol, followed by addition

of 3 M Hcl, and measuring the fluorescence at 500 nm, using excitation at 365 nm.

The inactivation assay is carried out as follows: ACE and various amounts of inhibitor in 50 mM potassium phosphate, 100 mM NaCL, pH 8.3 are incubated in a total volume of 100 µl. At various time points, a 5 µl aliquot of the reaction mixture is withdrawn and assayed for enzyme activity according to the procedure described above.

EXAMPLE 4

Collagenase A obtained from Sigma Chemical Co. (Catalog No. C0773, Type VII, 95 % protein) is employed in assay and inactivation studies.

2-furanacryloyl-L-leucylglycyl-L-prolyl-L- alanine (referred to herein as "FALGPA") is used for the assay of the synthetic substrate. The concentration was 0.05 mM in 50 mM tricine, 0.4 M NaCl, 10 mM $CaCl_2$, pH 7.5 (see Van Wart and Steinbrink, Anal. Biochem. 113, 356 (1981)).

The inactivation studies were carried out as described for enkephalinase and ACE.

EXAMPLE 5

The synthesis of the inhibitors of the invention which are collagenase inhibitors (i.e., dipeptide-analogs derivatized with a dipeptide or dipeptide ester) are carried out in a manner similar to that employed for the other analogs. To prepare the analogs derivatized with dipeptides, the methyl ester of the dipeptide to be added to the (e.g., pentanoic acid) dipeptide-analog is coupled to the analog using mixed anhydride and then the derivative of the invention is made by alkaline hydrolysis of the resulting methyl ester. To prepare the analogs derivatized with dipeptide esters, the alkyl ester of the dipeptide to be added to the (e.g., pentanoic acid) dipeptide-analog is simply coupled to the analog using mixed anhydride, yielding the ester derivative of the invention.

EXAMPLE 6

Synthesis of dimethyl benzylmalonate

To 100 ml of methanol at 0°C was added dropwise 4.5 ml of acetyl chloride and the reaction mixture was stirred for 5 min. The methanolic Hcl solution was then warmed to 23°C, 6 grams of benzylmalonic acid were added, and the solution was refluxed for 35 hours. After concentration by rotary evaporation, an oil was obtained which was taken up in 300 ml of ethyl acetate and washed and saturated sodium bicarbonate followed by brine. The organic layer was dried over $MgSO_4$, and concentrated to give 6.8 grams (100%) of the product.

^1H NMR ($CDCl_3$): delta 7.15-7.30 (m, 5H), 3.70 (s, 6H), 3.22 (d, 2H, J = 7.81 Hz); IR (neat) 1731 cm^{-1}

Benzylmalonic acid monomethyl ester

To 6.8 grams (0.0309 mmoles) of dimethyl malonate in 45 ml of methanol was added dropwise a solution of 1.24 grams (0.031 mmoles) of sodium hydroxide in 45 ml of methanol with stirring. The mixture was stirred at 23°C for 16 hours, then concentrated and taken up in 200 ml of water. The solution was titrated to pH 2.00 and the aqueous layer was extracted twice with 250 ml of ethylacetate. The combined organic layers were dried over $MgSO_4$, and concentrated in vacuo to afford 5.6 gr (87%) of the product as an oil.

^1H NMR ($CDCl_3$) delta 7.32-7.19 (m, 5H), 3.71 (s, 3H), 3.20 (m, 1H), 3.24 (m, 2H); IR 3246, 3030, 1736, 1714 cm^{-1}

3-phenyl-2-carbomethoxy-propionyl chloride

To 0.91 gms (4.375 mmoles) of benzylmalonic acid monomethyl ester in 55 µl of benzene, was added 55 µl of DMF and, dropwise, 477 µl (5.47 mmoles) of oxalyl chloride at 23°C, resulting in rapid gas evolution. After stirring for 30 min, the solution was concentrated, taken up in 20 ml THF, and evaporated once again to ensure removal of unreacted oxalyl chloride. Traces of solvent were removed

under vacuum, and the acid chloride was used immediately for the next step without further purification.

(R,S) methyl 2-benzyl-3-oxo-4-cyano-butanoate

To 210 mg (8.75 mmoles) of sodium hydride in 68 ml of anhydrous THF under N_2 was added a solution of 1.723 grams (8.75 mmoles) of t-butyldimethylsilyl cyanoacetate in 20 ml THF over 5 min., and the reaction was allowed to proceed for 15 min. The reaction mixture was cooled to -78°C and a solution of 3-phenyl-2-carbomethoxy-propionyl chloride (max. 4.375 mmoles) in 20 ml THF was added dropwise over a period of 15 min. After stirring at -78°C for 30 min, the mixture was warned to 23°C over 30 min and then quenched with 78 ml of 0.06N Hcl. The solution was extracted with ethyl acetate (300 ml, followed by another 100 ml), the organic layers combined, washed with brine, dried and concentrated. Two drops of triethylamine were added, and the mixture was purified by flash chromatography using 30% ethyl acetate in hexane. The major fractions of the product were combined and then further purified by flash chromatography using 0.5% methanol in chloroform to give 350 mg (37% starting from benzyl malonic acid monomethyl esters) as an oil. ^1H NMR ($CDCl_3$): delta 7.32-7.14 (m, 5H), 3.95 (t, 1H, J = 7.7 Hz), 3.48 (AB, q, 2H J = 19.7 Hz, diff. in freq.AB=56.2 Hz), 3.22 (d, 2H, J = 7.7 Hz); IR 2955, 1747, 1727 cm^{-1}

t-Butyldimethylsilyl (TBDMS) Cyanoacetate

To a solution of cyanoacetic acid (1.7 grams, 20 mmoles) and t-butyldimethylsilyl chloride (3.14 grams, 20 mmoles) in 22 ml of anhydrous ethyl acetate at 0°C and under N_2 was added 2.71 ml of triethylamine, resulting in the immediate precipitation of triethylamine hydrochloride. The reaction mixture was stirred at 0°C for 30 minutes and then allowed to warm to ambient temperature. The suspension was filtered, and the salt precipitate was washed with ethyl acetate (2 x 20 ml). The filtrates were

combined and concentrated to afford 3.55 grams (90%) of the TBDMS ester as a clear oil. ^1H NMR ($CDCl_3$): delta 3.47 (s, 2H), 0.96 (s, 9H), 0.32 (s, 6H); IR (neat) 3455, 2934, 2862, 2266, 1731, 1471 cm^{-1}.

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WHAT IS CLAIMED IS:

1. A compound of Formula X

$$NC-CHR_2-(C=O)-(CR_3R_4)z-(CR_5R_6)_x-CHR_9-CO-X_2 \quad X$$

wherein z is 0 or 1, wherein, when z is 0, the group $-(CR_3R_4)_z-(CR_5R_6)_x-$ is not in the compound and the group X_1- is bonded directly to the group $-(CHR_9)-$; x is 0 if z is 0 or is 0 or 1 if z is 1, wherein, when x is 0 and z is 1, the group $-(CR_5R_6)_x-$ is not in the compound and the group $-(CR_3R_4)-$ is bonded directly to the group $-(CHR_9)-$; R_3, R_4, R_5, and R_6 are independently hydrogen or alkyl of 1 to 3 carbon atoms; R_9 is benzyl, alkyl of 1 to 5 carbon atoms, or hydrogen; X_2 is joined to the -CO- in an amide linkage and is selected from the group consisting of glycine, N-methyl-glycine, N-benzyl-glycine, D-alanine, L-alanine, β-alanine, D-phenylalanine, L-phenylalanine, D-leucine, L-leucine, 3-amino propionic acid, D-proline, L-proline, and the group X_3-X_4, wherein X_3 is joined to the $-CHR_9-CO-$ group in an amide linkage and is selected from the group consisting of L-proline, L-alanine, L-valine, L-leucine, and L-O-methyltyrosine; when X_3 is L-proline or L-alanine, X_4 is selected from the group consisting of L-arginine, L-proline, L-leucine, L-alanine, L-hydroxyproline, and L-homoarginine; and when X_3 is L-valine, L-leucine, or L-O-methyltyrosine, X_4 is selected from the group consisting of glycine, L-alanine, and the alkyl esters of glycine and alanine, wherein the alkyl is of 1 - 5 carbons; provided that, if R_9 is benzyl, x is 0; if X_2 is X_3-X_4 and X_3 is L-proline or L-alanine, z is 1, x is 0, and R_9 is hydrogen or methyl; and if X_2 is X_3-X_4 and X_3 is L-leucine, L-valine or L-O-methyltyrosine, z is 1, x is 0 and R_9 is alkyl of 3 - 5 carbon atoms; and physiologically acceptable salts thereof.

2. A compound according to Claim 1 wherein R_2, R_3, and R_4 are hydrogen; and R_9 is benzyl.

3. A compound according to Claim 2 wherein X_2 is selected from the group consisting of glycine, N-methyl-glycine, D-alanine, L-alanine, L-phenylalanine, L-leucine, L-proline and 3-amino propionic acid.

4. A compound according to Claim 3 wherein the configuration at the carbon bound to R_9 is R.

5. A compound according to Claim 1 wherein R_2, R_3, R_4, R_5 and R_6 are hydrogen; R_9 is methyl or hydrogen; and X_2 is L-proline.

6. A compound according to Claim 5 wherein R_9 is methyl and the configuration at the carbon bound to R_9 is R.

7. A compound according to Claim 5 wherein R_9 is hydrogen.

8. A compound according to Claim 6 wherein x is 0.

9. A compound according to Claim 7 wherein x is 0.

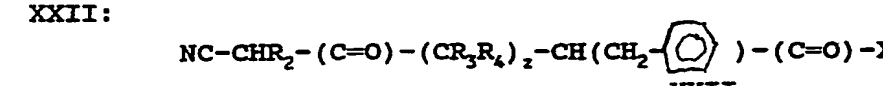
10. A compound according to Claim 1 wherein R_2, R_3, R_4, and R_9 are hydrogen; x is 0, and X_2 is X_3X_4, wherein X_3 is selected from the group consisting of L-proline and L-valine and X_4 is selected from the group consisting of L-arginine, L-proline, L-leucine, L-alanine, L-hydroxyproline, and L-homoarginine.

11. A compound according to Claim 1 wherein R_2, R_3, and R_4 are hydrogen; R_9 is isobutyl; x is 0; and X_2 is X_3X_4, wherein X_3 is selected from the group consisting of L-leucine, L-valine and L-O-methyltyrosine and X_4 is selected from the group consisting of glycine, L-alanine, and alkyl esters of glycine and L-alanine, wherein the alkyl group is of 1 - 5 carbon atoms.

12. A compound according to Claim 11 wherein X_4 is selected from glycine ethyl ester and L-alanine ethyl ester.

13. 2-benzyl-5-cyano-4-oxopentanoic acid substantially free of 3-benzyl-5-cyano-4-oxopentanoic acid.

14. A method of reducing pain in a mammal suffering therefrom comprising administering to said mammal a pain-reducing-effective amount of a compound of Formula XXII:



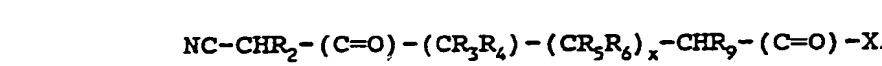
XXII,

wherein z is 0 or 1, wherein, if z is 0, the group $-(CR_3R_4)_z-$ is not present in the compound and X_1 is bonded directly to the group $-CH(CH_2-\bigcirc)-$; R_2, R_3, and R_4 are independently hydrogen or alkyl of 1 - 3 carbon atoms; and X_{12} is joined to the $-(C=O)-$ in an amide linkage and is selected from the group consisting of glycine, N-benzyl-glycine, L-alanine, D-alanine, L-phenylalanine, D-phenylalanine, L-leucine, D-leucine, and 3-amino propionic acid; or a pharmacologically acceptable salt thereof.

15. A method according to Claim 14 wherein the mammal to be treated is a human and, in the compound to be administered, R_2, R_3, and R_4 are hydrogen; and X_{12} is selected from the group consisting of glycine, D-alanine, L-alanine, L-phenylalanine, L-leucine, and 3-amino propionic acid.

16. A method according to Claim 15 wherein, in the compound to be administered, the configuration at the carbon bound to $-CR_3R_4-$ and a benzyl group is R.

17. A method for treating hypertension in a mammal suffering therefrom comprising administering to said mammal an antihypertensive-effective amount of a compound of Formula XV



XV

wherein R_2, R_3, R_4, R_5, and R_6 are independently hydrogen or alkyl of 1 to 3 carbon atoms; R_9 is benzyl, methyl or hydrogen; X_{15} is joined to the $-(C=O)-$ in an amide linkage and is selected from the group consisting of glycine, N-methyl-glycine, N-benzyl-glycine, D-alanine, L-alanine,

D-phenylalanine, L-phenylalanine, D-leucine, L-leucine, 3-amino propionic acid, D-proline and L-proline; and x is 0 or 1, wherein, when x is 0, the group $-(CR_5R_6)-$ is not in the compound, provided that, if R_9 is benzyl, x is 0; or a physiologically acceptable salt thereof.

18. A method according to Claim 17 wherein the mammal to be treated is a human and wherein, in the compound to be administered, R_2, R_3, and R_4 are hydrogen; R_9 is benzyl; and X_{15} is selected from the group consisting of glycine, N-methyl-glycine, L-alanine, L-phenylalanine, L-leucine, and L-proline.

19. A method according to Claim 18 wherein, in the compound to be administered, the configuration at the carbon bound to $-CR_3R_4-$ and a benzyl group is R.

20. A method according to Claim 19 wherein the mammal to be treated is a human and wherein, in the compound to be administered, R_2, R_3, R_4, R_5 and R_6 are hydrogen; R_9 is methyl or hydrogen; and X_{15} is L-proline.

21. A method according to Claim 20 wherein, in the compound to be administered, R_9 is methyl and the configuration at the carbon to which R_9 is bound is R.

22. A method according to Claim 21 wherein, in the compound to be administered, R_9 is hydrogen.

23. A method according to Claim 22 wherein, in the compound to be administered, x is 0.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/05902

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [1]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07C 255/00; C07D 207/08,207/46; A61K 37/02, 37/00

US Cl.: 514/19; 558/405,440,445; 260/998.2; 548/568; 530/337

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System [3]	Classification Symbols
U.S.	514/19; 558/405,440,445; 260/998.2; 548/568; 530/337

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	US, A, 2,437,906, (Bruson et al.) 16 March 1948, see entire document.	1-13
A	US, A, 3,529,009, (Funten et al.) 15 September 1970, see entire document.	1-13
A	US, A, 3,824,271, (Allen et al.) 16 July 1974, see entire document.	1-13
A	J. Med. Chem., Vol. 31, issued 1988, Plattner et al. "Renin Inhibitors Dipeptide analogues of angiotensin utilizing a structurally modified phenylalanine residue to impart proteolytic stability", pages 2277-2288, see page 2283; figure IV.	14-23

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
10 January 1991	15 MAR 1991
International Searching Authority [3]	**Signature of Authorized Officer [20]**
ISA/US	CARMEN PILI-CURTIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
A	Burger, "Medicinal Chemistry", published 27 June 1960, Interscience Publishers Inc. N.Y. pp. 565-601; see entire document.	14-23
A	Denkewalter et al., "Progress in Drug Research", published 1966; pages 510-512, see page 512.	14-23

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	J. Med. Chem., Vol. 30, issued 1987, Balis et al., "Renin Inhibitors Dipeptide analogues of angiotensin incorporating transition state non peptidic replacements of scissile", pages 1729-1737, see p. 1732.	14-23
A	J. Cardiovascular Pharmaology, Vol 10., suppl.,7 issued 1987, Haber et al., "Renin Inhibitors: A Search for Principles of Design"; pages 554-558, see p. 554 and 556.	14-23

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See paper #9.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.